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ORIGINAL PAPER

Assessing the Microbial Communities in Four Different Daqus by Using PCR-DGGE, PLFA, and Biolog Analyses

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Abstract

Daqu made from raw wheat, barley or pea is used as an inoculum for the fermentation of Chinese Baijiu. In this study, the microbial communities of four different types of Daqus (sauce-flavor Wuling Daqu, sauce and strong-flavor Baisha Daqu, strong-flavor Deshan Daqu, and light-flavor Niulanshan Daqu) were analyzed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), phospholipid fatty acid (PLFA) analysis, and Biolog EcoPlates analysis (Biolog). Clear differences were seen between the microbial communities of the four Daqus. PCR-DGGE showed differences in the number and brightness of bands between the Daqus, indicating the presence of unique bacterial species in Deshan Daqu, Wuling Daqu, and Niulanshan Daqu. *Lactobacillus sanfranciscensis*, *Bacillus thermoamylovorans*, and some unclassified bacteria were unique to Wuling Daqu, Deshan Daqu, and Niulanshan Daqu, respectively. Moreover, some bacterial species were observed in all four Daqus. A total of 26 PLFAs between C12 to C20 were detected from the four Daqus by PLFA analysis. Wuling Daqu had the highest total and fungal biomasses, Baisha Daqu had the highest bacterial biomass, and Niulanshan Daqu had the highest ratio of fungal biomass to bacterial biomass. The Biolog results indicated differences in the carbon source use and mode of the four Daqus, and also demonstrated that each Daqu had varying abilities to utilize different types of carbon sources. The cluster analysis of the three methods showed that the microbial communities of the four Daqus were different. This study also demonstrates the applicability of the three analytical methods in the evaluating of the microbial communities of Daqus.

Key words: Microbial community, Daqu, PCR-DGGE, PLFAs, Biolog

Introduction

The flavor characteristics of Chinese Baijiu, a traditional liquor, can be attributed to the unique craftsmanship employed during production, materials in different regions of China and different starters (Liu et al. 2018). Chinese Baijiu stems from cereals such as sorghum and wheat by complex fermentation processes using a naturally blended starter culture called Daqu, which is one of three different types of the starters used in Chinese Baijiu (Wang et al. 2018). Various microbes in Daqu are necessary for macromolecular hydrolysis and metabolism, which contributes to a large number of flavor compounds and precursors (He et al. 2019). Thus, Chinese Baijiu can be roughly divided into four main flavors: sauce-flavor, sauce and strong-flavor, strong-flavor, and light-flavor (Wang et al. 2017).

Daqu, which is produced from raw wheat, barley or peas, works as a saccharifying and fermenting agent to produce the liquor. Daqu can collect and enrich a variety of environmental microorganisms, enzymes, metabolites, and degradation products and it is also the determinative factor and power source for liquor fermentation (Li et al. 2015). Many intrinsic (the properties of the raw materials, the open fermentation environment, and the complexity of procedures) and extrinsic factors of Daqu production influence the richness and structure of the microbial communities and make it difficult to elucidate the exact specifications of the microbial communities in Daqu (Jin et al. 2017). Different types of Daqu can be

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distinguished based on their maximum incubation temperatures (Liu et al. 2018). A high-temperature Daqu is cultured at 60–70°C and it is mainly used for the sauce-flavor liquor production (Liu et al. 2018). A medium-temperature Daqu is formed at 50–60°C and it is mainly used for the strong-flavor liquor production. A low-temperature Daqu is heated to ≤ 50°C and it is mainly used for the light-flavor liquor production (Liu et al. 2018). Thus, Daqus with different microbial communities can form liquors with distinctive flavors. Moreover, a large number of liquor brewing enterprises countrywide in China, with their unique ecological environments and diverse manufacturing procedures result in the typical "home microbiota" with a large diversity of microorganisms in the Daqu (Zheng et al. 2011).

Several previous studies have been focused on investigating the microbial communities of Daqu, but relatively few have focused on the microbial communities of different Daqus that give rise to distinctive flavors. In the past, culture-dependent methods were the commonest approach to microbial profiling analysis. However, microorganisms identified using these methods amount to ≤ 10% of the total environmental microorganisms and do not reflect the actual microbial profile distribution within Daqu (Liu et al. 2017). Culture-independent methods such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis are highly useful to detect the whole microbial communities in Daqu samples (Muyzer et al. 1998; Ahmadsah et al. 2018). Phospholipid fatty acid (PLFA) analysis is considered to reflect the actual condition of the microbial communities because this analytical method is based on the extraction and quantification of phospholipids from all microorganisms in the sample; this method has been applied to Daqu (Jiang et al. 2018). The total concentration of PLFA can be used as an indicator of viable microbial biomass, hence it can help us to understand the microbial communities (Awad et al. 2018). The diversity of microbial communities can also be measured by Biolog EcoPlate analysis (Biolog), which provides comprehensive information on the metabolism of various microbes. The plates are useful in microbial community studies and are used widely to characterize bacterial communities in various fields, but no one has used them in Daqu research (Zeng et al. 2018). The profiling of the carbon source used by the microbial communities present in different Daqus can be performed readily using Biolog EcoPlates (Kumar et al. 2017).

In this study, we applied PCR-DGGE, PLFA analysis, and Biolog plates to examine in-depth the microbial communities in four different aroma-style Daqus. The aim of this study was to analyze the microbial communities in different Daqus and to compare the composition of the microbial communities in different Daqus by using three different analyses.

Experimental

Materials and Methods

Sample collection and preparation. In May 2016, four types of matured Daqu blocks (having been matured for 6 months): Wuling Daqu, Baisha Daqu, Deshan Daqu, and Niulanshan Daqu were respectively obtained from Hunan Wuling Spirits Co., Ltd., Baisha Spirits Industry Co., Ltd., Hunan Deshan Spirits Industry Co., Ltd., and Niulanshan Distillery, Beijing Shunxin Agriculture Co., Ltd. Sampling data are shown in Table I. Three parallel samples were collected from the top, middle, and bottom of the storeroom, and were transferred into sterile bags and stored at –20°C. After milling and mixing, samples were stored at 4°C.

DNA extraction and PCR amplification. Based on the methodology of Milanović et al. (2018), total DNA was extracted from the treated samples. Genomic DNA was extracted using a PowerSoil DNA Isolation Kit (Mo-Bio, Carlsbad, CA, USA) per the method described in the operating instructions. The extracted genomic DNA was detected by 0.6% agarose gel electrophoresis and stored at –80°C (Gan et al. 2017; Liang et al. 2018). Template DNA (2 µl) was used to amplify the bacterial 16S rRNA gene using the primers F338GC (5'-CGCCCGCCGCGCGCGGC GG GCGG GGCGGGGGCACGGGGGGACTCCTACGGGA GGCAGCAG-3') and R518 (5'-ATTACCGCGGCTG CTGG-3') in 50 µl reaction mixtures containing 1 µl of each primer (10 µM), 4 µl of 2.5 mM dNTPs, 5 µL 10 × of PCR buffer with $MgCl_2$ and 1.25 U polymerase. The

Table I
Samples of four typical Daqus of Chinese spirits.

Name	Flavour type	Highest temperature inside the Daqu pile (°C)	Region (city and geographic coordinates)
Wuling	Sauce-flavour	65	Changde, Hunan (29°05'N, 111°39'E)
Baisha	Sauce- and strong-flavour	60	Changsha, Changsha (28°11'N, 112°58'E)
Deshan	Strong-flavour	55	Changde, Hunan (29°05'N, 111°39'E)
Niulanshan	Light-flavour	50	Beijing (39°56'N, 116°20'E)

amplification conditions were: 95°C for 5 min, 94°C for 5 min, 65–55°C for 30 s, 72°C for 3 min, 32 cycles (the annealing temperature was decreased by 0.5°C after each cycle); and 94°C for 1 min, 55°C for 50 s, 72°C for 3 min, 32 cycles, with a final elongation at 72°C for 10 min. The amplified products were verified by 0.8% agarose gel electrophoresis (Ahmadsah et al. 2018).

DGGE analysis. The resultant amplicons were analyzed by DGGE using a D-Code TM Universal Mutation Detection System (Bio-Rad, USA). 45 µl of each amplified product was separated on an 8% (w/v) polyacrylamide gel in 1 × TAE buffer at 60°C, with a denaturing gradient from 40% to 60% (100% denaturant corresponds to 7 M urea and 30% formamide) at 20 V for 20 min and 100 V for 15 h. Gels were stained with ethidium bromide for 15 min, visualized using a gel imaging and analysis system, digitized by the Image processing software Quantity One 1-D, and analyzed by the unweighted pair group method with arithmetic averages (UPGMA). The coefficient of similarity (Cs) indicated community structure similarity between different samples (Sha et al. 2018). The major bands were excised, and eluted DNA was re-amplified as described above using primers without a GC-clamp. The PCR products were purified and sent to the Beijing Genomics Institute (Beijing, China) for clone sequencing. Sequence information was analyzed by aligning the results with sequences in GenBank using BLAST (Bligh et al. 1959; Wang et al. 2018).

PLFA extraction and analysis. PLFAs were extracted using a modified Bligh and Dyer procedure (Jiang et al. 2018). 5 g of Daqu and 30 ml of the Bligh and Dyer extraction buffer were placed into a 50 ml centrifuge tube. The samples were homogenized and incubated in the dark overnight at 4°C. Then, 7.5 ml of chloroform and 7.5 ml of phosphate buffer were added to the mixture; the tube was shaken and incubated overnight at 4°C. The following day, the samples were separated by centrifugation at 3000 × g for 10 min. The bottom layer was transferred by passage through a filter into a flask wrapped in tin foil, after which the solvent was evaporated with N_2. The dried extracts were suspended in 300 µl of chloroform and sequentially eluted from an activated silicic acid column with 10 ml of chloroform, and a mixture of 10 ml of chloroform and 10 ml of methanol (Holmstrup et al. 2018). The phospholipids were collected in test tubes, mixed with 500 µl of methanol, 500 µl of toluene and 1 ml of 0.2 M KOH, and incubated at 37°C for 15 min. Following this, 300 µl of 1 M acetic acid, 2 ml of n-hexane-chloroform mixture, and 2 ml of ultrafiltrated water were added to the samples, which were mixed for 1 min and allowed to stand for 1 h, resulting in a two-phase mixture. The upper organic phase was transferred to a clean tube and evaporated under N_2 flow (Hsiao et al. 2018). The extracts were dissolved in n-hexane-chloroform mixture, spiked with methyl nondecanoate (19:0), and analyzed by gas chromatography-mass spectrometry (GC-MS). The operating conditions were: 50°C for 1 min, increased at 12°C/min to 180°C and held for 2 min; increased at 6°C/min to 220°C and held for 2 min; increased at 15°C/min to 240°C and held for 1 min; increased at 15°C/min to 260°C and held for 15 min. The injector temperature was 230°C and the GC-MS interphase temperature was 280°C. Helium was used as carrier gas (Kang et al. 2018; Li et al. 2019).

Community-level physiological profiling. The metabolic functional diversity of Daqu microbial communities (community-level physiological profiling) was analyzed by applying the Biolog EcoPlates method (Capó-Bauçà et al. 2019). Sample (10 g) was suspended in 200 ml of sterile water and shaken for 30 min at 150 rpm. After a 2-min settling time, 1 ml of supernatant was diluted in 9 ml of sterile water. The suspension was diluted (1:1000) in sterile water. Diluent (150 µl) was added into the wells of Biolog EcoPlates, which contained three replicates of 31 carbon sources and three blanks (water) wells. The microplates were incubated at 25°C. Optical density (OD) was measured every 12 h for 480 h at 590 nm. The metabolic activity of microorganisms was determined by average well color development (AWCD) (Li et al. 2019).

Results and Discussion

Bacterial communities revealed by DGGE. At least 20 visible bands, constituting the current community structures, were seen for each sample. The number and brightness of the bands varied between the four Daqus, indicating clear differences in their microbial communities. Comparing the fingerprints of the four Daqus, Wuling Daqu, Baisha Daqu, and Niulanshan Daqu had both unique bands and bands observed in all samples. These bands, including those designated a-m (see Fig. 1A), were sequenced for the homology alignment using the NCBI database. Bacteria identified from the bands were classified as *Firmicutes*, including *Thermoactinomycetaceae, Bacillaceae*, *Weissella*, and *Lactobacillaceae*, which were predominant in the samples. Some bacteria were unclassified.

Sequence alignment with a ≥ 95% cut-off revealed highly diversified bacterial communities in the four samples (Table II). *Lactobacillus sanfranciscensis* was unique to Wuling Daqu, and *Bacillus thermoamylovorans* was only detected in Deshan Daqu. Four unclassified bacteria were detected only in Niulanshan Daqu. The genus *Lactobacillus* was reported as the predominant lactic acid bacteria in the sauce flavored Daqus (Tang et al. 2017). Zou et al. (2018) found that *Bacillus*

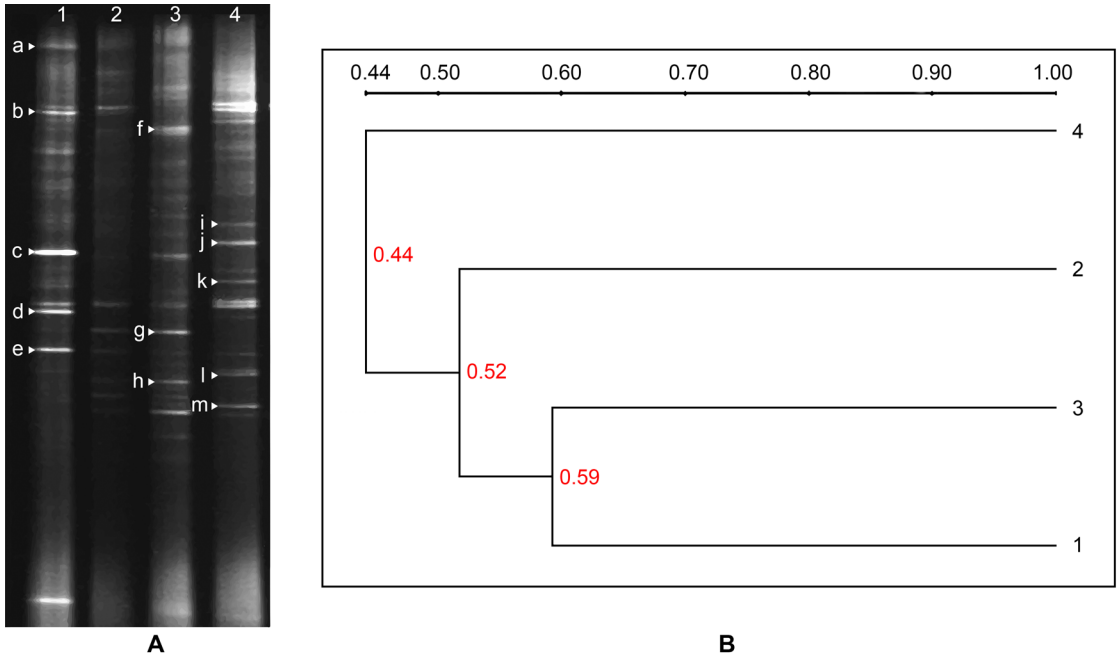

Fig. 1. PCR-DGGE profiles (A) and clustering analysis (B) of the bacterial communities in four typical Daqu samples. (a-m represent the specific bands excised; 1–4 represent Wuling Daqu, Baisha Daqu, Deshan Daqu, and Niulanshan Daqu, respectively.

thermoamylovorans was present in the strong-flavored Daqus. In contrast, *Pediococcus pentosaceus* could be detected in Wuling Daqu and Deshan Daqu, *Weissella confuse* were enriched in all Daqus other than Deshan Daqu, and *Pediococcus acidilactici* was present in Baisha Daqu and Deshan Daqu. Consistent with the previous research results, *Pediococcus* has been reported in both the sauce-flavored Daqu and the strong-flavored Daqu (Tang et al. 2017; Zou et al. 2018), *Weissella* was obtained in the sauce-flavored, sauce and strong-flavor and light-flavored Daqu (Zhang et al. 2014; Yunita et al. 2018), and *Pediococcus* was found in the strong-flavored Daqu (Zou et al. 2018).

Multivariate analysis of DGGE profiles. UPGMA cluster analysis was used to examine relationships among the four Daqus (Yunita et al. 2018). Comparisons by the UPGMA cluster analysis (Fig. 1B) using the Dice correlation coefficient showed that Wuling

Table II
Summary of the identification of bands in Fig. 1.

Band No.[a]	Related GenBank sequence	Closest relatives (accession no.)	Identity (%)[b]
a	MN857671	Uncultured bacterium (AB441615.1)	100
b	MN857663	*Weissella confuse* (GU049413.1)	99
c	MN857670	*Pediococcus pentosaceus* (AB481102.1)	100
d	MN857669	*Lactobacillus sanfranciscensis* (EU350220.1)	99
e	MN857662	Uncultured *Lactobacillus* sp. (FJ982856.1)	100
f	MN857666	Uncultured bacterium (AB441567.1)	100
g	MN857665	*Pediococcus acidilactic*i (FJ751795.1)	99
h	MN857667	*Bacillus thermoamylovorans* (GU067470.1)	99
i	MN857672	Uncultured bacterium (FJ235654.1)	100
j	MN857673	Uncultured bacterium (GQ076030.1)	96
k	MN857664	Uncultured bacterium (GQ505035.1)	100
l	MN857661	Uncultured *Lactobacillus* sp. (GQ999780.1)	98
m	MN857668	*Thermoactinomyces sanguinis* (AJ251778.1)	95

[a] Bands are numbered according to Fig. 1.
[b] Identity represents the sequence identity (%) compared with that in the GenBank database.

Table III
The concentration of the PLFAs in different Daqu samples.

PLFA (nmol/g dry matter)	Wuling Daqu	Baisha Daqu	Deshan Daqu	Niulanshan Daqu
A11:0	0	0	0	105.36
A13:0	327.32	297.50	153.95	99.80
15:00	294.13	475.82	189.31	24.35
Me14:0	133.53	0	0	217.31
I14:0	117.82	0	0	54.73
I15:0	0	0	0	34.62
A15:0	102.96	0	0	44.73
16:1W9Z	121.91	0	83.49	0
16:00	4.15	194.31	4.00	5.45
I16:0	95.05	220.59	0	0
A16:0	113.83	166.87	2.86	142.79
17:00	112.01	250.34	61.09	0
Cy17:0	0	0	76.79	0
18:3W6,9,12t	216.81	0	1.58	0
18:3W3,6,9zzz	0	0	66.79	0
18:2W6.9tt	1.76	3.03	0	2.66
18:2W6.9zz	234.20	30.46	0	186.88
18:2W6.8zz	0	0	0	51.43
18:2W7.10tt	0	0	0	315.07
18:2W5.8tt	248.88	0	3.18	0
18:1W9t	5.89	8.97	14.18	7.36
18:1W10t	45.45	0	0	0
18:1W9z	0	0	0	68.80
18:00	24.92	68.60	14.55	26.90
Cy18:0	149.68	0	0	0
20:00	152.12	0	0	0

Daqu and Deshan Daqu grouped first, and then clustered with Baisha Daqu and Niulanshan Daqu in order, indicating that the bacterial communities in the four Daqus were significantly different.

PLFA profiles of Daqus. The PLFA analysis method has often been used to study environmental microbial communities and has occasionally been used in the study of the strong-flavor and light-flavor Daqu (Zhang et al. 2016). Here, 26 PLFAs with chain lengths ranging from C12 to C20 were identified in total in the four samples (Table III). The patterns of abundance varied between the four different samples. The number of PLFAs detected in Wuling Daqu was 19, more than in the other three Daqus. PLFAs 18:1ω10t, cy18:0, and 20:00 were only detected in Wuling Daqu, and the dominant PLFAs a13:0, 15:0, 18:2ω5.8tt, and 18:2ω6.9zz in Wuling Daqu accounted for 44.14% of the total PLFAs in that Daqu. Moreover, the total biomass and the fungal biomass were the highest in Wuling Daqu. Sixteen types of PLFA were detected in Niulanshan Daqu, among which PLFAs a11:0, I15:0, 18:2ω6.8zz, 18:2ω7.10tt, and 18:1ω9z were unique to Niulanshan Daqu, and PLFAs me14:0, a16:0, 18:2ω7.10tt, and 18:2ω6.9zz predominated, accounting for 62.10% of the PLFAs in that Daqu. The ratio of fungi to bacteria was the highest in Niulanshan Daqu, reaching 4.23, but the bacterial biomass was the lowest. PLFA cy17:0 was only detected in Deshan Daqu, and its dominant PLFAs were a13:0 and 15:0, accounting for 51.40% of the 10 types of PLFA detected in this Daqu. Deshan Daqu had the lowest total biomass of the four Daqus. Similarly, only 10 types of PLFA were detected in Baisha Daqu, and the dominant PLFAs a13:0, 15:0, 16:0, 17:0, and I16:0 accounted for 72.49% of the total PLFAs. Among the Daqus, the bacterial biomass was highest in Baisha Daqu and the fungal biomass was the lowest (Fig. 2). The fungal biomass of Wuling Daqu was 20.95-times that of Baisha Daqu, and the bacterial biomass of Baisha Daqu was 10.92-times that of Niulanshan Daqu (Fig. 2A). The ratios of fungi to bacteria in Niulanshan Daqu and Wuling Daqu were >1 (Fig. 2B). The differences in total biomass, bacterial biomass, fungal biomass, and the ratio of fungal to bacterial biomass between samples in this study demonstrate that different Daqus have unique microbial communities (Zhang et al. 2016).

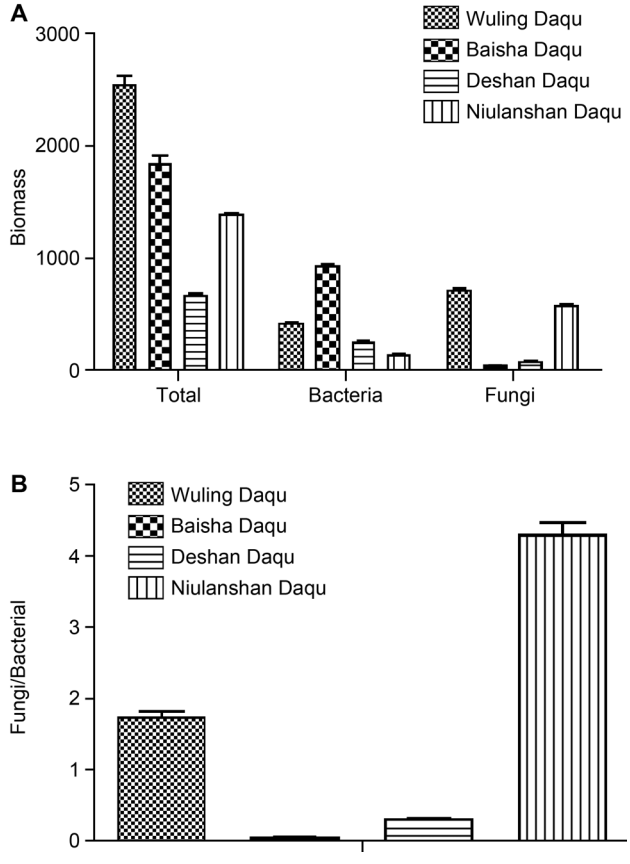

Fig. 2. Total biomass, bacterial biomass, fungi biomass (A) and the ratio of fungi biomass to bacteria biomass (B) of Daqu samples.

The results of principal component analysis (PCA) were shown in Fig. 3A. The first principal component

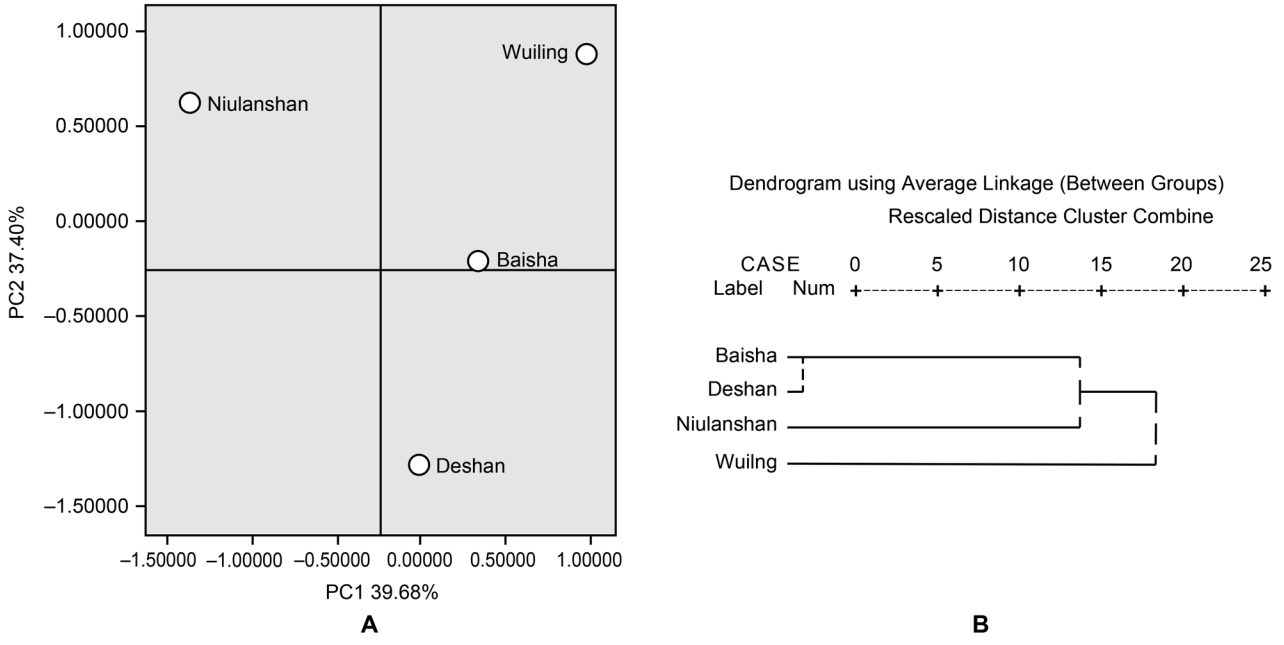

Fig. 3. Principal component analysis (PCA) showing variations in the PLFA pattern in different types of Daqu (A); clustering analysis (B) of the four Daqus on PLFAs content.

(PC1) and the second principal component (PC2) of the absolute PLFA abundances accounted for 39.68% and 37.40% of the variance respectively; thus, PC1 and PC2 captured 77.08% of the total data variability. Wuling Daqu had a positive correlation with PC1 and PC2, and Niulanshan Daqu was positively correlated with PC2. Deshan Daqu was negatively correlated with PC2, and PC1 had less influence on Deshan Daqu, while PC2 had almost no effect on Baisha Daqu. The profiles of microbial communities of the four Daqus were statistically analyzed via the Euclidean distance and cluster analysis (Fig. 3B). Based on the Euclidean distance datasets for all samples, Baisha Daqu and Deshan Daqu formed a cluster and then were classified with Niulanshan Daqu and Wuling Daqu in order.

Average well color development. The dynamic patterns of AWCD values in Biolog EcoPlate analyses are shown in Fig. 4. Table IV listed the 31 carbon sources used. The AWCD value increased significantly for the first 144 h, then stabilized. The highest utilization of carbon sources was the microbes in Niulanshan Daqu, followed by Wuling Daqu, Deshan Daqu, and Baisha Daqu (Fig. 4A). Based on the principle of microbial metabolism of the three major nutrients, the 31 carbon source substrates were divided into five categories: monosaccharides and their derivatives; disaccharides and polysaccharides; amino acids and their derivatives; fatty acids and lipids; and metabolic intermediates and secondary metabolites (Gillis et al. 2019). For monosaccharides and their derivatives, disaccharides and polysaccharides, and metabolic intermediates and secondary metabolites, Niulanshan Daqu was the best among the four samples, while Baisha Daqu was the worst (Fig. 4B, C, F). For amino acid substrates and their derivatives, there was no significant difference between Wuling Daqu and Niulanshan Daqu, which were both higher than Deshan Daqu and Baisha Daqu (Fig. 4D). For fatty acids and lipids, the utilization capacities of Wuling Daqu, Niulanshan Daqu, and Deshan Daqu were all relatively similar, and higher than that of Baisha Daqu (Fig. 4E). In general, the metabolic rates of carbon sources were determined by calculating a single value (AWCD) at a single time point, which enabled a clear comparison of the microbial communities (Kumar et al. 2017).

PCA converted 31 carbon sources into a few comprehensive variables to reflect the overall characteristics of the microbial use of carbon sources (Fig. 5A). PC1 and PC2 accounted for 53.022% and 29.002% of the variance and together captured 82.024% of the total data variability. PC1 was affected by 13 types of carbon sources, including disaccharides, polysaccharides, and monosaccharides and their derivatives. PC2 was affected by 13 types of carbon sources, including monosaccharides and their derivatives, metabolic intermediates, secondary metabolites, and amino acids and their derivatives (Table IV). Niulanshan Daqu had a positive correlation with PC1, and Deshan Daqu was positively correlated with PC2. Baisha Daqu was negatively correlated with PC1 and PC2, while PC1 and PC2 had almost no effect on Wuling Daqu. In cluster analysis, Baisha Daqu and Deshan Daqu formed a cluster, and were then classified with Baisha Daqu, followed by Niulanshan Daqu (Fig. 5B).

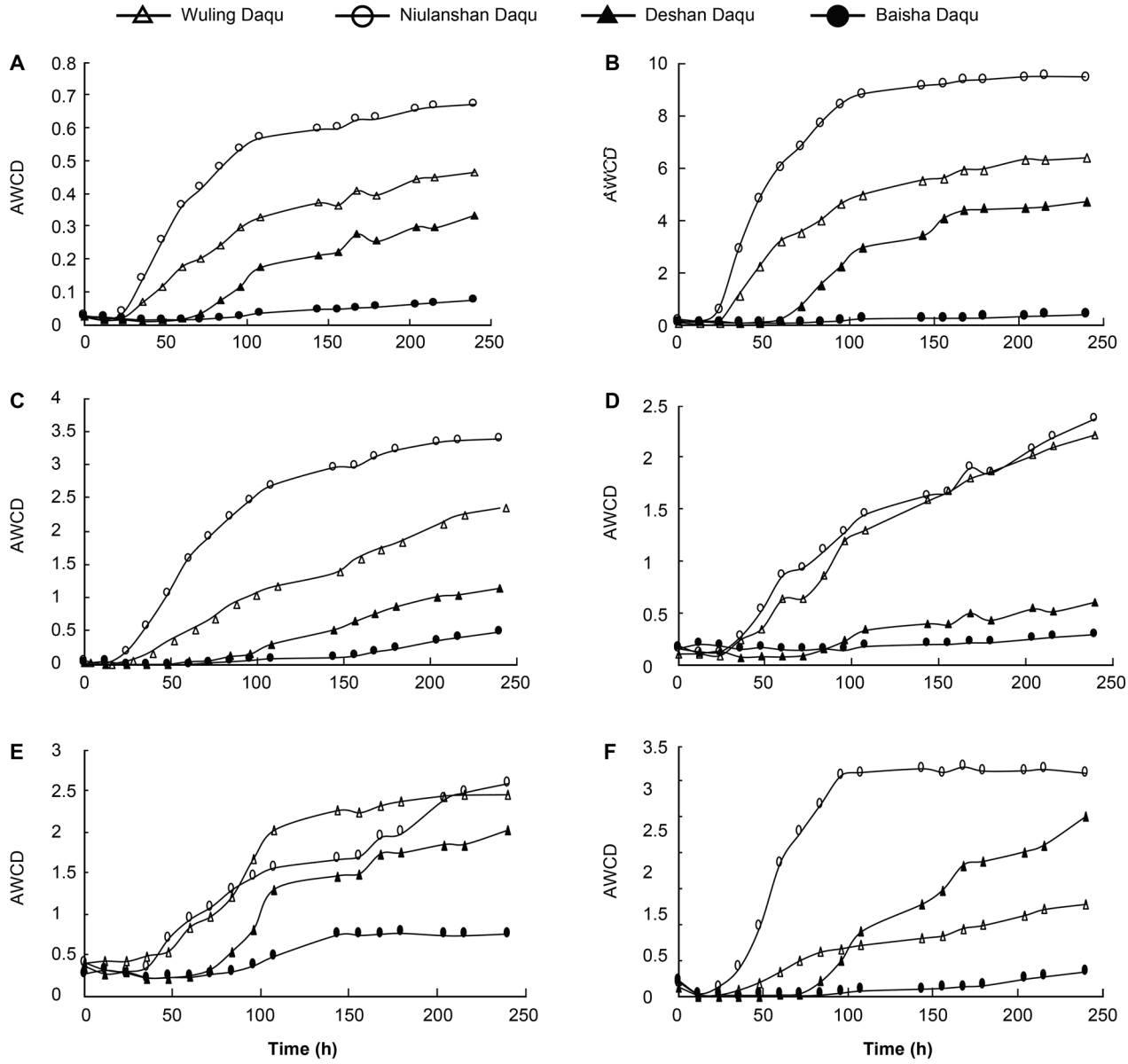

Fig. 4. The AWCD of five types of carbon sources in four Daqus communities, including all carbon sources (A), monosaccharides and their derivatives (B), disaccharides and polysaccharides (C), amino acid substrate and its derivatives (D), fatty acids and lipids (E), and metabolites and secondary metabolites (F).

Several environmental factors, including the regional climate and microorganisms in the air, jointly may influence the microorganism composition in Daqus (Li et al. 2015). The manufacture of Daqu is carried out in an open environment, which allows various kinds of microorganisms from the air to colonize the Daqu. The air contains microorganisms that colonize soil, water bodies, plant surfaces, rocks and buildings, released, for example, by wind and water flow. In turn, air microbiota can be deposited back to surfaces on the ground via dry and wet deposition processes (Polymenakou 2012). The microbial communities will differ depending on the environment. Temperature is a limiting factor for cell activity in the air, and airborne microbes can also suffer from desiccation (Polymenakou 2012). Because of varying ambient temperature and humidity, the growth of microorganisms differs between regions. The northern city of Beijing, which is the birthplace of Niulanshan Daqu, has a very different climate to the regions from which the other Daqus used in this study originated. Beijing has a typical continental temperate monsoon subhumid climate. The distribution of precipitation is very uneven; annual precipitation is concentrated in summer (July and August) (Liu et al. 2009). The annual average temperature is 10–12°C, the annual average precipitation is 470–660 mm, and the annual average relative humidity is 51% (Liu et al. 2014). Wuling Daqu and Deshan Daqu are produced in Changde, which is in a humid subtropical climate zone. The annual average temperature is generally 15–22°C,

Table IV
Comparison of the carbon utilization of different samples.

Well	Carbon Sources	Wuling Daqu	Baisha Daqu	Deshan Daqu	Niulanshan Daqu
A2	β-Methyl-D-glucoside	0.559	0	0.001	1.445
A3	D-Galactonic acid-γ-Lactone	0.526	0.026	1.199	1.028
A4	L-Arginine	0.383	0.019	0.316	0.063
B1	Pyruvic acid Methyl ester	0.759	0	0.383	0.444
B2	D-Xylose	1.115	0.025	0.067	1.500
B3	D-Galacturonic acid	1.484	0	0.754	1.391
B4	L-Asparagine	0.146	0.033	0.035	0.919
C1	Tween 40	0.872	0.356	0.399	0.399
C2	i-Erythritol	0.113	0.002	0.21	0.263
C3	2-Hydroxy benzoic acid	0.004	0	0.176	0
C4	L-Phenylalanine	0.085	0.121	0.099	0.132
D1	Tween 80	0.558	0.251	0.8	1.037
D2	D-Mannitol	0.845	0.008	0.399	1.789
D3	4-Hydroxy benzoic acid	0.019	0.020	0.302	0.076
D4	L-Serine	1.129	0.031	0.049	0.626
E1	α-Cyclodextrin	0.001	0.048	0	0.007
E2	N-Acetyl-D-glucosamine	0.927	0.171	0.146	1.844
E3	γ-Hydroxybutyric acid	0.118	0.102	0.139	0.042
E4	L-Threonine	0.031	0	0	0.019667
F1	Glycogen	0.192	0	0.143	0.163
F2	D-Glucosaminic acid	0.298	0	0.967	0.023
F3	Itaconic acid	0	0.044	0	0
F4	Glucose-L-glutamic acid	0.021	0.009	0	0.132
G1	D-Cellobiose G2	0.920	0.122	0.422	1.538
G2	Glucose-1-phosphate	0.109	0.033	0	1.255
G3	a-Ketobutyric acid	0	0	0.004	0
G4	Phenylethylamine	0.001	0	0.534	0
H1	a-D-Lactose	0.612	0.013	0.189	1.404
H2	D,L-a-Glycerol phosphate	0.162	0.009	0.243	0.275
H3	D-Malic acid	0.349	0.009	0.431	0.944
H4	Putrescine	0.293	0.053	0.133	0.639

and the annual average precipitation is 1200–1900 mm, the annual average relative humidity is 87% (Huang et al. 2014). Baisha Daqu is produced in Changsha, which is also located in a humid subtropical climate zone (Liu et al. 2011; Wu et al. 2019). The annual average temperature is generally 16–17°C, the annual average precipitation is 1359–1553 mm, and the annual average relative humidity is 81% (Yao et al. 2018). The climatic differences between these Daqu producing regions lead to changes in the microbial communities in the environment, which in turn affects the microbial communities in the Daqu. Cluster analysis of PCR-DGGE results confirmed this finding.

Varied production processes of Daqu also lead to different physical and chemical properties, resulting in differences in the microbial species present (Zheng et al. 2011). Temperature is an important technological parameter that determines the microbial communities in Daqu by affecting the growth and death rates of microorganisms (Li et al. 2015). Wuling Daqu, Deshan Daqu, and Niulanshan Daqu belong to high-temperature, medium-temperature and low-temperature Daqu, respectively, while Baisha Daqu has a fermentation temperature between medium temperature and high temperature. The specific temperature during Daqu production may result in distinct compositions of microbes (Shanqimuge et al. 2015). The results of this study (cluster analysis of PCR-DGGE and Biolog results) agree with this suggestion.

To our knowledge, this is the first study to apply PCR-DGGE, PLFA analysis, and Biolog to simultaneously analyze four typical Daqus (Deshan Daqu, Baisha

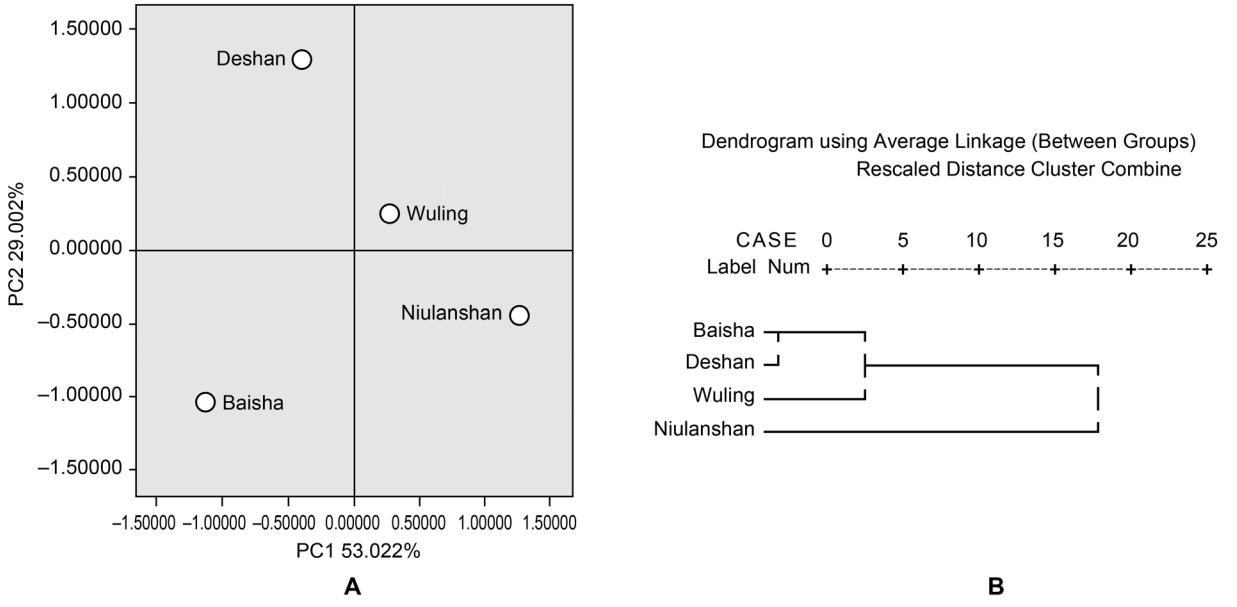

Fig. 5. Principal component analysis (A) and clustering analysis (B) based carbon source utilization patterns of microbial communities.

Daqu, Wuling Daqu, and Niulanshan Daqu), revealing clear differences in their microbial communities. Studies of microbial communities in Daqus are difficult and involve a series of methodological challenges, including the inability to culture the vast majority of microorganisms present in Daqus (Borymski et al. 2018). The results obtained here using the respective methods were not in perfect agreement (Xue et al. 2008). This, however, may not be surprising since each method analyzes a different feature of the Daqu microbial communities and is associated with its advantages and disadvantages in determining the microbial diversity and community structure. The results obtained with Biolog favor the rapidly growing and most active microbe over the slow-growing ones that exist in Daqus. However, this method can be used to compare the functionality and biodiversity of the microbial communities (Borymski et al. 2018). The PLFA analysis determines the number of living microbial cells because phospholipids exist in all living cells but they are rapidly degraded after cell death. Unlike Biolog, the PLFA analysis does not require the culture of microorganisms and analyzes all extractable PLFA. However, the results obtained by this method depend on the extraction efficiency of PLFA and many environmental factors (Borymski et al. 2018). The PCR-based molecular methods are more robust and allow for significantly higher resolution. 16S rRNA gene-targeted PCR-DGGE is reliable, reproducible, fast and inexpensive (Gupta et al. 2019). Multiple samples can be analyzed in one run, allowing for simultaneous profile comparison, corresponding to microbial communities (Xue et al. 2008; Sułowicz et al. 2016). The major shortcomings of PCR-DGGE method are related to PCR itself. The DNA isolation efficiency may vary depending on the method employed. In addition, depending on the DNA fragment mobility, one band may represent multiple species or sequences from the same species that can result in more than one band (Xue et al. 2008; Sułowicz et al. 2016). In our study, clustering results derived from the three methods and PCA of the PLFA and Biolog data showed that the microbial communities of the four Daqus were different. PCA results from the PLFA and Biolog data allowed clear, visual differentiation of the four Daqus (Fig. 3A and 5A); these findings may be ascribed to the different production temperatures and environments of the Daqu producing regions. However, the clustering result from PLFA analysis may be due to methodological problems, and this needs further analysis (Fig. 1B, 3B, and 5B). However, one of the roots of the experimental design of this paper was to analyze the microbial communities of four Daqus from different perspectives to understand and differentiate the microbial communities as comprehensively as possible.

Conclusions

This study analyzed the microbial communities of four different types of Daqu by using PCR-DGGE, PLFA, and Biolog, which provided useful information for the study of the fermentation of Chinese Baijiu. The microbial communities of Daqus of four different flavor types from different climatic and environmental sources were different, and these differences may also be influenced by the unique fermentation temperature of each Daqu. Three commonly used analytical methods illustrate this difference in different ways. From our

data, it is concluded that the microbial communities of Daqus from similar regions and fermentation temperatures are similar. Further investigations need to be conducted to obtain more detailed information on the contribution of microbial communities of Daqus to the final formation of the unique aromas of Chinese Baijiu.

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Authors' Contributions

Zuming Li and Zhihui Bai conceived and designed the experiments; Wenying Li performed the experiments; Wenying Li, Yuxi Ling, Zhihui Bai and Zuming Li analyzed the data; Yuxi Ling wrote the paper; Tong Tong and Qian Li gave important suggestions; Zuming Li and Zhihui Bai revised the manuscript. Guijun Wang, Jiahao Chen and Yuguang Wang provided samples for the experiments.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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